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Fuels and Chemicals from Biomass

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Chapter 2

Enzymes in Lignocellulosic Biomass Conversion

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Advances in enzymes and lignocellulosic biomass processing are
necessary to lower the cost of fuels and chemicals production
from biomass. Recent developments in lignocellulosic biomass
conversion enzymology and process technology are reviewed.
Current problems of these multi-enzymes based complex
processes, economic assessment, regulatory issues, strategies for
development of improved enzymes and processes, and directions
of future research are discussed. Results of our endeavor to
develop novel enzymes for biomass conversion are presented.

Currently, more than one billion gallons of ethanol are produced annually in the United
States, with approximately 95% derived from fermentation of corn starch (*1*). Enzymes
play an important part in the conversion of corn starch to glucose that is then fermented
to ethanol by yeast. In fact, application of amylases in starch conversion is a great
example of the successful use of enzymes in biotechnology. With increased attention
to clean air and oxygenates for fuels, opportunities exist for rapid expansion of the fuel
ethanol industry. Various lignocellulosic biomass such as agricultural residues, wood,
municipal solid wastes and wastes from pulp and paper industry have potential to serve
as low cost and abundant feedstocks for production of fuel ethanol or chemicals. Right
now, the use of lignocellulosic biomass to produce fuel ethanol represents significant
technical and economic challenges, and its success depends largely on the development
of highly efficient and cost-effective biocatalysts for conversion of pretreated biomass
to fermentable sugars. In this article, we describe briefly current knowledge on the
application of enzymes in various lignocellulosic biomass conversion.

Lignocellulosic Biomass

Lignocellulosic biomass includes various agricultural residues (straws, hulls, stems,
stalks), deciduous and coniferous woods, municipal solid wastes (MSW, paper,

cardboard, yard trash, wood products), waste from pulp and paper industry and herbaceous energy crops. The compositions of these materials vary. The major component is cellulose (35-50%), followed by hemicellulose (20-35%) and lignin (10-25%). Proteins, oils and ash make up the remaining fraction of lignocellulosic biomass (*2*). The structure of these materials is very complex and native biomass is resistant to an enzymatic hydrolysis. In the current model of the structure of lignocellulose, cellulose fibers are embedded in a lignin-polysaccharide matrix. Xylan may play a significant role in the structural integrity of cell walls by both covalent and non-covalent associations (*3*). The pretreatment of lignocellulosic biomass is crucial before enzymatic hydrolysis. Various pre-treatment options are available now to fractionate, solubilize, hydrolyze and separate cellulose, hemicellulose and lignin components (*4-7*). These include steam explosion, dilute acid treatment, concentrated acid treatment, alkaline treatment, treatment with SO_2, treatment with hydrogen peroxide, ammonia fiber explosion, and organic solvent treatments. In each option, the biomass is treated to reduce its size and open its structure. Pretreatment usually hydrolyzes hemicellulose to its sugars (xylose, L- arabinose, and other sugars) that are water soluble (*4*).   The residue contains cellulose and lignin. The lignin can be extracted with solvents such as ethanol, butanol or formic acid. Alternatively, hydrolysis of cellulose with lignin present makes water-soluble sugars and the residues are lignin plus unreacted materials. The use of SO_2 as a catalyst during steam pretreatment resulted in the enzymatic accessibility of cellulose and enhanced recovery of the hemicellulose derived sugars (*8*).   Steam pretreatment at 200-210°C with the addition of 1% SO_2 (w/w) was superior to other forms of pretreatment of willow (*9*).   A glucose yield of 95%, based on the glycan available in the raw material, was achieved. A summary of various pretreatment options is given in Table I. Recently, supercritical carbon dioxide explosion was found to be very effective for pretreatment of cellulosic materials before enzymatic hydrolysis (*10*). The sequential steps for production of fuels and chemicals from lignocellulosic biomass involve feedstock preparation, pretreatment, fractionation, enzyme production, hydrolysis, fermentation, product recovery, and waste treatment.   The pretreatment of lignocellulosic biomass is an expensive procedure with respect to cost and energy.

Cellulose conversion

Cellulose is a linear polymer of 8,000-12,000 D-glucose units linked by 1,4-ß-D-glucosidic bonds. The enzyme system for the conversion of cellulose to glucose comprises endo-1, 4-ß-glucanase (EC 3.2.1.4), exo-1, 4-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21). Cellulolytic enzymes with ß-glucosidase act sequentially and cooperatively to degrade crystalline cellulose to glucose. Endoglucanase acts in a random fashion on the regions of low crystallinity of the cellulosic fiber whereas exoglucanase removes cellobiose (ß-1, 4 glucose dimer) units from the non-reducing ends of cellulose chains. Synergism between these two enzymes is attributed to the *endo-exo* form of cooperativity and has been studied extensively between cellulases in the degradation of cellulose   in *Trichoderma reesei* (*11*). ß-Glucosidase hydrolyzes cellobiose and in some cases cellooligosaccharides to glucose. The enzyme is generally responsible for the regulation of the whole cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose as both endoglucanase and

Table I. Methods for pretreatment of lignocellulosic biomass

Method	Example
Thermo-mechanical	Grinding, milling, shearing, extruder.
Autohydrolysis	Steam pressure, steam explosion, supercritical carbon dioxide explosion.
Acid Treatment	Dilute acid (H_2SO_4, HCl), concentrated acid (H_2SO_4, HCl), acetic acid.
Alkali treatment	Sodium hydroxide, ammonia, alkaline hydrogen peroxide.
Organic solvents treatment	Methanol, ethanol, butanol, phenol.

cellobiohydrolase activities are often inhibited by cellobiose (*12-14*). Thus, β-glucosidase not only produces glucose from cellobiose but also reduces cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently. However, like ß-glucanases, most ß-glucosidases are subject to end-product (glucose) inhibition. The kinetics of the enzymatic hydrolysis of cellulose including adsorption, inactivation and inhibition of enzymes have been studied extensively (*15*). For a complete hydrolysis of cellulose to glucose, the enzyme system must contain the three enzymes in right proportions.

Product inhibition, thermal inactivation, substrate inhibition, low product yield and high cost of cellulase are some barriers to commercial development of the enzymatic hydrolysis of cellulose. Many microorganisms are cellulolytic. However, only two microorganisms (*Trichoderma* and *Aspergillus*) have been studied extensively for cellulase. There is an increasing demand for the development of thermostable, environmentally compatible, product and substrate tolerant cellulase with increased specificity and activity for application in the conversion of cellulose to glucose in the fuel ethanol industry. Thermostable cellulases offer certain advantages such as higher reaction rate, increased product formation, less microbial contamination, longer shelf-life, easier purification and better yield.

In our work, forty-eight yeast strains belonging to the genera *Candida*, *Debaryomyces, Kluyveromyces* and *Pichia* (obtained from the ARS Culture Collection, Peoria, IL) were screened for production of extracellular glucose tolerant and thermophilic ß-glucosidase activity using p-nitrophenyl-ß-D-glucoside as substrate (*16*). Enzymes from 15 yeast strains showed very high glucose tolerance (< 50% inhibition at 30%, w/v glucose). The optimal temperatures and pH for these ß-glucosidase activities varied from 30 to 65°C and pH 4.5 to 6.5. The ß-glucosidase from *D. yamadae* Y-11714 showed highest optimal temperature at 65°C followed by *C. chilensis* Y-17141(60°C) and *K. marxianus* Y-1195 (60°C). The optimal pH of these three enzyme preparations were 6.5, 6.0 and 6.5, respectively. The temperature and pH profiles of β-glucosidases from *C. chilensis* Y-17141, *D. yamadae* Y-11714 and *K. marxianus* Y-1195 are shown in Figure 1. The β-glucosidases from all these yeast strains hydrolyzed cellobiose. Novel glucose tolerance and thermoactivity found in the enzyme preparations from *D. yamadae, K. marxianus* and *C. chilensis* are desired
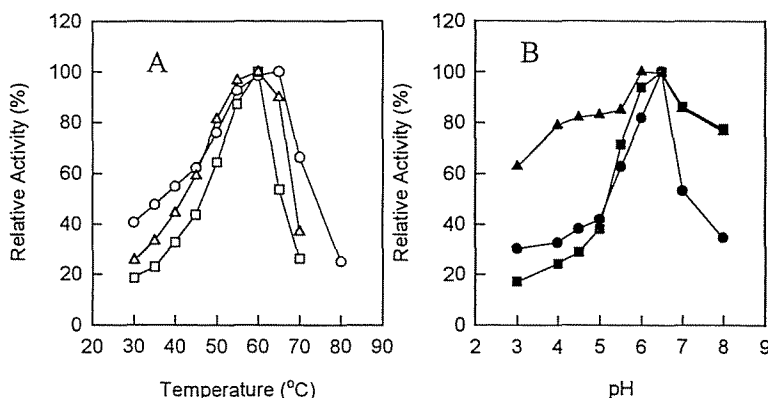
Figure 1. Effect of temperature (A) and pH (B) on the activity of extracellular β-glucosidases from *Candida chilensis* Y-17141, *Debaryomyces yamadae* Y-11714 and *Kluyveromyces marxianus* Y-1195. Each value is average of duplicate experiments for each enzyme preparation. Buffers (0.1 M) used: acetate (pH 3.0-5.5) and phosphate (6.0-8.0). Symbols: (□,■), *C. chilensis* Y-17141; (○,●), *D. yamadae* Y-11714; (△,▲), *K. marxianus* Y-1195.

Table II. Biochemical characteristics of thermostable ß-glucosidase from *Aureobasidium pullulans* NRRL Y-12974 (*17*)

Specific activity	315 U/mg protein
Molecular weight	340,000 (2 subunits), glycoprotein
Optimum temperature	75-80°C
Optimum pH	4.5
Specificity	Hydrolyzes cellobiose and cello-
oligosaccharide	
Half-life (crude enzyme)	24 h at 80°C; 72 h at 75°C
K_m value (mM)	
pNPßG (at pH 4.5, 75°C)	1.17
Cellobiose (at pH 4.5, 75°C)	1.00
Metal ion requirement	None
Substrate inhibition	
pNPßG (20 mM)	No inhibition
Cellobiose (6 %, w/v)	No inhibition
Inhibition by glucose	Competitive (K_i=5.65 mM)

attributes of a β-glucosidase suitable for industrial application for enzymatic hydrolysis of cellulose to glucose. We have purified and characterized a highly thermophilic β-glucosidase from a color variant strain of *Aureobasidium pullulans* (*17*). Some properties of this enzyme are summarized in Table II.

The cellulose hydrolysis step is a significant component of the total production cost of ethanol from wood (*18*).   Achieving a high glucose yield is necessary (>85% theoretical) at high substrate loading (>10% w/v) over short residence times (< 4 days). It was shown that simultaneous saccharification (hydrolysis) of cellulose to glucose and fermentation of glucose to ethanol (SSF) improve the kinetics and economics of biomass conversion by reducing accumulation of hydrolysis products that are inhibitory to cellulase and β-glucosidase, reducing the contamination risk because of the presence of ethanol, and reducing the capital equipment requirements (*19*). An important drawback of SSF is that the reaction has to operate at a compromised temperature of around 30°C instead of enzyme optimum temperature of 45-50°C. Enzyme recycling, by ultrafiltration of the hydrolyzate, can reduce the net enzyme requirement and thus lower costs (*20*). Hinman et al. (*21*) reported that a preliminary estimate of the cost of ethanol production for SSF technology based on wood-to-ethanol process is $ 1.22/gal of which the wood cost is $ 0. 459/gal. Wright et al. (*22*) evaluated a separate fungal enzyme hydrolysis and fermentation process for converting lignocellulose to ethanol. The cellulase enzyme was produced by the fungal mutant *Trichoderma* Rut C-30 (the first mutant with greatly increased β-glucosidase activity) in a fed batch production system that is the single most expensive operation in the process. The conversion of lignocellulosic biomass to fermentable sugars requires the addition of complex enzyme mixtures tailored for the process and parallel reuse and recycle the enzymes until the cost of enzymes comes down. Enzyme recycling can increase the rates and yields of hydrolysis, reduce the net enzyme requirements and thus lower costs (*23*). The first step in cellulose hydrolysis is considered as the adsorption of cellulase onto cellulosic substrate.   As the cellulose hydrolysis proceeds, the adsorbed enzymes (endo- and exo-glucanase components) are gradually released in the reaction mixture.   The β-glucosidase does not adsorb onto the substrate. These enzymes can be recovered and reused by contacting the hydrolyzate with the fresh substrate. However, the amount of enzyme recovered is limited because some enzymes remain attached to the residual substrate and some enzymes are thermally inactivated during hydrolysis. It has been shown that several substrates containing a high proportion of lignin result in the poor recovery of cellulase (*24*).

Gusakov et al. (*25*) found that cellolignin was completely converted to glucose by cellulase from *T. viride* and *A. foetidus*. Cellolignin was an industrial residue obtained during the production of furfural from wood and corn cobs when pretreated by dilute H_2SO_4 at elevated temperature. The concentration of glucose in the hydrolyzate reached 4-5.5%, cellulose conversion being not less than 80%. Kinetic analysis of cellolignin hydrolysis, using a mathematical model of the process, has shown that, with product inhibition, nonspecific adsorption of cellulase onto lignin and substrate induced inactivation seem to affect negatively the hydrolysis efficiency. Borchert and Buchholz (*26*) investigated the enzymatic hydrolysis of different cellulosic materials (straw, potato pulp, sugar beet pulp) with respect to reactor design. The kinetics was studied including enzyme adsorption, inhibition, and inactivation. The results suggest the use of reactors with plug flow characteristics to achieve high substrate and product concentrations and to avoid back-mixing to limit the effect of product inhibition. For efficient use of cellulases, a reactor with semipermeable hollow fiber or an ultrafilter membrane was used and this allowed cellulases to escape end-product inhibition (*27-30*). A totally

integrated biotechnology of rice straw conversion into ethanol was reported (*31*). It dealt with (i) ethanol refining of rice straw to segregate cellulose from pentose sugars and lignin, (ii) preparation of highly active mixed cellulase enzymes, (iii) a novel reactor system allowing rapid product formation involving enzymatic hydrolysis of cellulose to sugars followed by microbial conversion of the later into ethanol and its simultaneous flash separation employing a programmed recompression of ethanol vapors and condensation, and (iv) concentration of ethanol via alternative approaches.

In direct microbial conversion of lignocellulosic biomass into ethanol that could simplify the ethanol production process from these materials and reduce ethanol production costs, *Clostridium thermocellum*, a thermoanaerobe was used for enzyme production, hydrolysis and glucose fermentation (*32*). Cofermentation with *C. thermosaccharolyticum* simultaneously converted the hemicellulosic sugars to ethanol. However, the formations of by-products such as acetic acid and low ethanol tolerance are some drawbacks of the system. Several recent reviews have dealt with the molecular biology of cellulose degradation, cellulolytic enzyme systems, and the structure and function of various domains found in the enzymes involved (*33-36*).

Hemicellulose conversion

Hemicelluloses are heterogeneous polymers of pentoses (xylose and L-arabinose), hexoses (mannose) and sugar acids. Xylans, major hemicelluloses of many plant materials, are heteropolysaccharides with a homopolymeric backbone chain of 1,4-linked β-D-xylopyranose units. Besides xylose, xylans may contain L-arabinose, D-glucuronic acid or its 4-o-methyl ether, and acetic, p-coumaric, and ferulic acids.

The total hydrolysis of xylan requires endo β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), and several accessory enzyme activities such as α-L-arabinosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.1), acetyl xylan esterase (EC 3.2.1.6), feruloyl esterase and p-coumaroyl esterase which are necessary for hydrolyzing various substituted xylans. The endo-xylanase randomly attacks the main chains of xylans and β-xylosidase hydrolyzes xylooligosaccharides to xylose. The α-L-arabinosidase and α-glucuronidase remove the arabinose and 4-O-methyl glucuronic acid substituents, respectively from the xylan backbone. The esterases hydrolyze the ester linkages between xylose units of the xylan and acetic acid (acetyl xylan esterase) or between arabinose side chain residues and phenolic acids such as ferulic acid (feruloyl esterase) and p-coumaric acid (p-coumaroyl esterase). Synergistic action of depolymerizing and side-group cleaving enzymes has been proved using acetylated xylan as substrate (*37*). Bachmann and McCarthy (*38*) reported significant synergistic interaction between endo-xylanase, β-xylosidase, α-L-arabinofuranosidase, and acetyl xylan esterase enzymes of the thermophilic actinomycete *Thermomonospora fusca*. Many xylanases do not cleave glycosidic bonds between xylose units which are substituted. The side chains must be cleaved before the xylan backbone can be completely hydrolyzed (*39*). On the other hand, several accessory enzymes only remove side-chains from xylooligosaccharides. These enzymes require xylanases to hydrolyze hemicellulose partially before side-chains can be cleaved (*40*). Although the structure of xylan is more complex than cellulose and requires several different enzymes with different specificities for a complete hydrolysis, the polysaccharide does not form tightly

packed crystalline structures and is thus more accessible to enzymatic hydrolysis (41). The yeast-like fungus *Aureobasidium* is a promising source of xylanase (MW 20 kDa) with an exceptionally high specific activity (2100 U/mg protein) (42). Xylanase represented nearly half the total extracellular protein, with a yield of up to 0.3 g of xylanase per liter (43). A few recent reviews have dealt with the multiplicity, structure and function of microbial xylanases, and molecular biology of xylan degradation (3, 44, 45).

The utilization of hemicellulosic sugars is essential for efficient conversion of lignocellulose to ethanol. The commercial exploitation of the pentose fermenting yeasts for ethanol production from xylose is restricted mainly by their low ethanol tolerance, slow rates of fermentation, difficulty to control the rate of oxygen supply at the optimal level plus sensitivity to microbial inhibitors, particularly those liberated during pretreatment and hydrolysis of lignocellulosic substrates (46, 47). Xylose can also be converted to xylulose using the enzyme xylose isomerase and traditional yeasts can ferment xylulose to ethanol (48, 49). Xylose can be easily converted into xylitol, a potentially attractive sweetening agent by a variety of microorganisms (yeasts, fungi and bacteria) (50).

Lignin conversion

Lignin is a long chain heterogeneous polymer composed largely of phenylpropane units most commonly linked by ether bonds. It effectively protects the woody plants against microbial attack and only a few organisms including rot-fungi and some bacteria can degrade it (51). The conversion of cellulose and hemicellulose to fuels and chemicals will generate lignin as a by-product that can be burned to provide heat and electricity, converted to low-molecular weight chemicals, and used in the manufacture of various polymeric materials. As lignin makes up 15-25% in some lignocellulosic biomass, the selling price of lignin has a very large impact on ethanol price (18).

In recent years, removal of lignin from lignin-carbohydrate complex (LCC) has received much attention because of potential application in pulp and paper industry. The lignin barrier can be disrupted by a variety of pretreatments rendering the cellulose and hemicellulose more susceptible to enzymatic attack (52). There are many papers about microbial breakdowns of lignin, the enzymes and the pathways (53-56). The degradation of lignin by the basidiomycete *Phanerochaete chrysosporium* is catalyzed by extracellular peroxidases (lignin peroxidase, Lip and manganese peroxidase, MnP) in a H_2O_2-dependent process (57, 58). However, due to extreme complexity of the problem, a vast amount of research needs to be done to understand all the factors involved in lignin biodegradation process (59).

Screening for enzymes with targeted properties

The cost of the enzymes for enzymatic hydrolysis of cellulosic biomass is clearly the critical parameter from an economic point of view. Most of the industrial enzymes are produced by organisms isolated from natural sources by a labor intensive, unpredictable classical screening, strain selection, medium optimization for over production, fermentation and recovery process development. Screening of naturally occurring

microorganisms still may be the best way to obtain new strains and/or enzymes for commercial applications (*60*).   Fundamental tasks and strategies for commercial development of an enzyme from natural sources are shown in Figure 2.   Recombinant DNA technology and protein engineering have also proven as effective means of production of industrial enzymes (*61*).   The marketing of all enzymes is subject to a

Screening for microorganisms
⇓
Culture selection
⇓
Fermentation studies (preliminary)
⇓
Isolation, purification and final characterization
⇓
Evaluation
⇓
Toxicology
⇓
Regulatory agency
⇓
Improvement of fermentation and recovery process
development
⇓
Product formulation
⇓
Marketing

Figure 2.   Strategies for commercial development of an enzyme

variety of Federal laws and regulations. The generally recognized as safe (GRAS) status of an industrial enzyme depends on the source of its origin. Federal laws, regulations and policies that have an impact on industrial enzymes have been reviewed by Fordham and Block (*62*).

Concluding remarks

The industrial enzyme market approaches approximately one billion US dollars annually. Several enzymes have already become commodity chemicals for many industrial application purposes such as in the production of various corn syrups and sweeteners and fuel ethanol from starch. Right now, the market for the enzymes involved in various lignocellulosic biomass conversion is limited and depends entirely on their use in the conversion of various lignocellulosic feedstocks to fermentable sugars for the subsequent production of fuel alcohols and value-added chemicals. Currently, cellulolytic enzymes are expensive and their hydrolysis rates are very slow.   The development of an environmentally compatible highly efficient enzyme system free from product and substrate inhibitions for conversion of various pretreated agricultural residues to glucose

is very important for use of these materials for production of fuel alcohol. The market for these enzymes will expand rapidly if certain properties of them can be improved and if these enzymes are made available for biomass conversion at a competitive price like starch degrading enzymes. On the other hand, the development of a very efficient substrate pretreatment that increases the susceptibility of crystalline cellulose to enzymatic hydrolysis significantly will lower the cost of producing ethanol from lignocellulosic biomass.

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